20-hydroxyecdysone induces apoptosis in the labial gland of *Manduca sexta*

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Abstract: The labial glands of *Manduca sexta* die over 5 days during larva to pupa metamorphosis. This cell death is presumably triggered by endocrine cues. The mechanisms by which steroid hormones induce apoptosis, however, are poorly understood. To investigate the role that the insect molting hormone, 20-hydroxyecdysone (20HE), plays in apoptosis of larval structures, we injected animals with exogenous 20HE. Since metamorphosing *Manduca* larvae have high titers of 20HE in the hemolymph, we surgically removed the prothoracic glands, which secrete 20HE, in order to create hormone-free abdomens. Labial glands from whole animals were used to establish the baseline levels of apoptosis during metamorphosis. Lysosomes play a critical role in the degradation of insect tissues (salivary gland, intersegmental muscle, and fat body) and mammalian tissues (mammary gland, prostate gland, and uterus). Apoptosis was monitored using acid phosphatase activity and histochemistry to monitor lysosomes and TUNEL to detect DNA fragmentation. Glands that were exposed to 20HE displayed an increase in the number of lysosomes, movement of lysosomes, and activation of acid phosphatase compared to controls. Glands from abdomens that received a single injection of hormone demonstrated levels of apoptosis that were comparable to glands from abdomens that were subjected to multiple 20HE injections. Our results suggest that 20HE triggers apoptosis of the labial glands and that its continuous administration is not necessary to kill the cells.

Key words: 20-hydroxyecdysone, apoptosis, lysosomes, *Manduca*, labial gland, TUNEL

Abbreviations: (20HE) 20-hydroxyecdysone, (TUNEL) terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling, (AP) acid phosphatase.

Introduction

Apoptosis is readily recognizable in insects as larval tissues degenerate in order to make way for adult tissues (Miller 1950; Finlayson 1956). Insect tissues provide a preparation that is apoptosis-enriched as well as ease of experimentation. Metamorphosis is an instructive context in which to study apoptosis (Lockshin & Williams 1965a). One striking aspect of metamorphosis is the need for coordinated action by many tissues. For example, the epidermis, which produces the cuticle, and the nervous system, which produces the behavior the insect needs to extricate itself from the old cuticle,

must both be ready to molt at the same time, and any lack of coordination is likely to result in the death of the insect (Fahrbach 1997). The apoptosis of insect tissues during metamorphosis occurs at specific times in postembryonic life. The precise mechanism by which a cell is committed to and undergoes apoptosis remains unknown. The steroid hormone 20-hydroxyecdysone (20HE) regulates the timing of naturally occurring apoptosis in insects (Schwartz & Truman 1982; Truman & Schwartz 1984; Bennett & Truman 1985).

Manduca sexta, the tobacco hornworm, is an ideal model system for the study of apoptosis. Several tissues in *Manduca* undergo apoptosis during development. For example, at pupation the abdominal appendages used by the caterpillar for walking, the prolegs, disappear. This loss of peripheral structures is followed by the death of some of the motor neurons that terminated on the proleg muscles (Weeks & Truman 1985). The deaths of neurons at the end of larval life and adult abdominal ganglia after eclosion occur by apoptosis (Ewer et al. 1998). The prothoracic glands, which synthesize and secrete 20HE, initiate apoptosis during the pupa to adult metamorphosis (Dai & Gilbert 1997). *Manduca* muscles, like the dorsal external oblique 1 muscle, also die by apoptosis (Hegstrom et al. 1998). In many instances the death of these tissues can be blocked by protein synthesis inhibitors, suggesting that de novo protein synthesis is required (Weeks et al. 1993). The labial gland of the tobacco hornworm undergoes apoptosis during larval to pupal metamorphosis (Jochová et al. 1997a). This gland is much bigger and easier to isolate than the aforementioned *Manduca* tissues. The large size of the labial gland facilitates the execution of histochemical and microscopic techniques.

The labial gland (a homologue of the silk gland of *Bombyx mori* and the salivary gland of *Drosophila*) dies in 5 days during the larva to pupa transformation. The paired epithelial labial gland is a secretory gland that is approximately 0.2 mm in diameter, 17 cm long, and consists of a single layer of gigantic cells (100 x 100 x 40 m; Jochová et al. 1997a). The entire gland dies, except for the anterior duct, which differentiates into the labial gland in the adult moth. The labial gland provides a valuable system to study the mechanisms that regulate apoptosis since the death of the tissue is synchronous and involves nearly the entire tissue. Consequently, a substantial amount of homogeneous dead cells can be studied uncontaminated by living cells, which is not possible in vertebrate systems. Since apoptosis in the labial gland occurs over 5 days, we can isolate glands at different stages of development and sequentially study differences in the levels of cell death. Previously, we have shown by metabolic measurements that the levels of energy resources and second messengers are adequate during the earlier phases of cell death in the labial gland (Halaby et al. 1994).

Lysosomal activation is an integral part of apoptosis in some systems, including insect tissues during metamorphosis and degenerating mammalian tissues. The salivary glands of the blowfly, *Calliphora vomitoria*, and *Drosophila* undergoe a cell death that involves autophagic digestion by lysosomes (Bowen et al. 1996; Jones & Bowen 1993). Acid phosphatase has been used as the marker enzyme for lysosomes and a marker for apoptosis. Zakeri et al. (1994) demonstrated that the interdigital regions of normal mouse limbs displayed positive labeling for acid phosphatase by histochemistry. Acid phosphatase activity is augmented and lysosomes degrade the following tissues during apoptosis in mammals: mammary gland, prostate gland, ovary, and uterus (Helminen & Ericsson 1971; Searle et al. 1973; Verma 1983; Sensibar et al. 1990; Kasuya 1997). Here, we demonstrate that the movement of lysosomes, activation of lysosomal enzymes, and detection of single-stranded DNA breaks in dying labial gland cells are regulated by 20HE.

Materials & Methods

Rearing of Animals

Manduca sexta larvae were purchased from Carolina Biological Supply Company (Burlington, NC), grown in individual compartments at 25 °C with a 12 h photoperiod, and fed an artificial hornworm diet (Carolina). The larval to pupal metamorphosis was first detectable as the larvae underwent the initiation of wandering on day 0. This includes the cessation of feeding, exposing of the aorta, and seeking a place to burrow (Dominick & Truman 1985). Larvae were staged in terms of days prior to or after wandering.

Ligations

At day 0, *Manduca* larvae have relatively high levels of endogenous 20HE in the hemolymph (Bollenbacher et al. 1981). Consequently, it is difficult to ascertain whether alterations in cell death parameters are due to endogenous or exogenous 20HE. To circumvent this problem, we created essentially 20HE-free abdomens. The relatively large size of *Manduca* full grown fifth instar larvae (approximately 12 g) facilitates surgical and endocrine manipulations. The only known sources of 20HE in *Manduca* are the prothoracic glands, which are located in the first thoracic segment. Day 0 larvae were anesthetized on ice for 20 min, ligated around the first abdominal segment, using dental floss, and the anterior body was severed to remove the prothoracic glands. The wounds were sealed with Krazy Glue (Borden, Columbus, OH). Isolated abdomens were left untouched overnight to allow sufficient time for the endogenous 20HE levels to decline.

20HE Injections

Abdomens received either a single injection or one injection every 24 h of either 50 µg of 20HE or an equal volume of 10% ethanol (vehicle) prior to dissection of the labial glands. Each injection was placed in a different abdominal segment to avoid excessive damage to one site. Incubation times ranged from 0-120 h. The 20HE concentration in the isolated abdomens was between 2.2-2.9 x 10^{-5} M, which are physiological concentrations of the hormone (Bollenbacher et al. 1981).

Tissue Collection

Animals were anesthetized on ice for 10 min and the labial glands dissected. The anterior ducts, which do not undergo apoptosis, were not included in the experiments. Glands were fixed in 4% paraformaldehyde at 4°C overnight, frozen in Tissue Tek OCT (Miles, Elkhart, IN), and cut as 5 (m sections onto poly-L-lysine coated slides (Sigma).

Acid Phosphatase Assays

To visualize lysosomes and to monitor lysosomal enzyme activity we examined the marker enzyme of lysosomes, acid phosphatase (Pelletier & Novikoff 1972). Lysosomes were localized from slides of frozen sections using a histochemical acid phosphatase (AP) assay (Sigma, St. Louis, MO) as previously described (Halaby et al. 1994) with the exception that all incubations were performed at room temperature to accommodate insect tissues. Labial glands from whole animals undergoing metamorphosis as well as from isolated abdomens were used. The presence of AP was indicated by red focal precipitates, which were resolved by light microscopy.

A biochemical AP assay (Sigma) was performed by homogenizing glands in 0.5 ml of 0.9% NaCl and clarifying homogenates by centrifugation for 5 min at room temperature. The reaction mixture (0.5 ml of p-nitrophenyl phosphate (substrate), 0.5 ml of 90 mM citrate buffer, pH 4.8, and 0.1 ml of homogenate) was incubated for 30 min at room temperature, and the reaction was terminated by the addition of 5 ml of 0.1 N NaOH. In alkali, liberated p-nitrophenol was measured spectrophotometrically at 410 nm. This assay was used to assess the lysosomal enzyme activity of labial glands that were obtained from intact and ligated animals.

DNA Fragmentation

DNA fragmentation was assessed in frozen sections by a TUNEL method using the ApopTag (In Situ Apoptosis Detection Kit (Intergen,

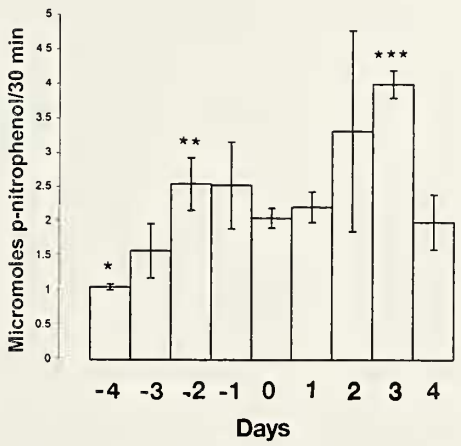

Fig 1. Acid Phosphatase Activity in Labial Glands during Metamorphosis. Labial glands were isolated from intact animals at various stages of development and the biochemical acid phosphatase (AP) assay was performed as described in Materials & Methods. The ages, representing days during the final larval stage, at dissection are indicated on the x-axis. Day 0 marks the beginning of larval to pupal metamorphosis. The values represent means of at least three independent experiments ± SEM. Asterisks indicate values significantly different from day -3: *, $p < 0.02$; **, $p < 0.04$; and ***, $p < 0.004$. Student's *t*-test was used for determination of statistical significance. Total AP activity is expressed on the y-axis as micromoles of p-nitrophenol (the product of the reaction catalyzed by AP) released per 30 min.

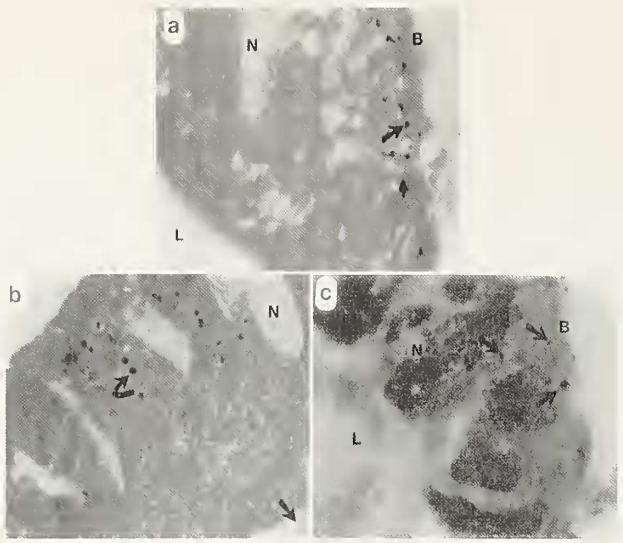

Fig. 2

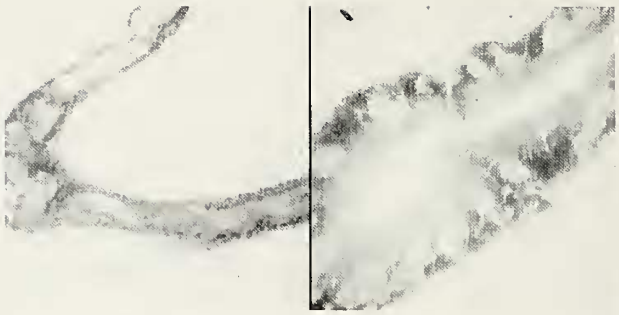

Fig. 4

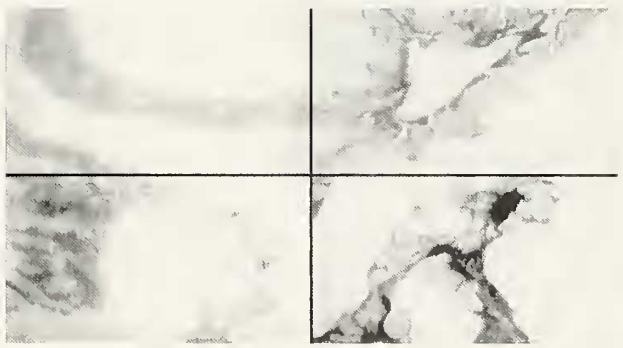

Fig. 5

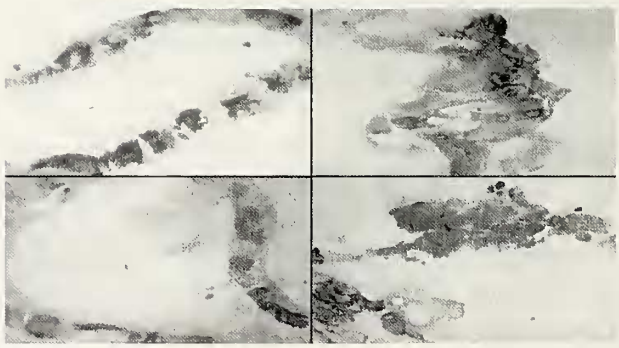

Fig. 6

Fig. 2. Histochemical Localization of Acid Phosphatase in Labial Glands during Metamorphosis. Glands were isolated from whole animals undergoing metamorphosis. (a) day -1 gland. (b) day 0 gland. (c) day 3 gland. Lysosomes were visualized using a histochemical AP assay. The presence of AP (arrows) is indicated by red focal precipitates (days -1 and 0) or diffuse staining (day 3), which were resolved by light microscopy. The lysosomes are restricted to basolateral regions of the cell on day -1. Lysosomes begin to migrate from basolateral areas towards apical, lumenal regions on day 0. By day 3 the lysosomes have increased in number, are located throughout the cytoplasm, and have migrated into the lumen. Lumen (L). Nuclei (N) appear intact. Basolateral surfaces (B). Magnification: 1,000X. Microscope: compound.

Fig. 4. Effect of a Single Exposure of 20HE on Lysosomes. Animals were ligated at day 0. Isolated abdomens received a single injection of 20HE or vehicle and glands were processed for the histochemical AP assay after 120 h. 20HE increased the number of lysosomes (red stain; right panel) and cell death in experimental glands compared to control glands. This suggests that a single exposure of the glands to 20HE may be sufficient to trigger apoptosis. Magnification: 100X. Microscope: compound.

Fig. 5. DNA Fragmentation during Metamorphosis. Glands were isolated from day 0 (a) and day 4 (b) metamorphosing whole animals. DNA fragmentation was assessed by TUNEL as described in Materials & Methods. Weak TUNEL staining was detected at day 0 (a). Remnant nuclei persisted which displayed intense TUNEL staining on day 4 compared to day 0 glands. Nuclei (N). Magnification: 100X. Microscope: compound.

Fig. 6. Effect of 20HE on DNA Fragmentation. Abdomens were injected with 20HE or vehicle. Some abdomens received one injection (lower panels) while others received multiple injections (one every 24 h; upper panels). In either case glands were assayed at 120 h for DNA fragmentation by TUNEL. Arrows indicate positive labeling of DNA single-strand breaks. A single administration of 20HE (lower right panel) induced a level of DNA fragmentation that was comparable to glands that were exposed to multiple treatments of 20HE (upper right panel). 20HE-treated glands displayed fewer nuclei, however the TUNEL labeling of the remaining nuclei was more intense than that observed in control glands (left panels). Magnification: 100X. Microscope: compound.

Purchase, NY). To digest the sections, Oncor protein digesting enzyme (20 µg/mL) was applied to the specimens for 15 min at room temperature followed by four washes in distilled H_2O for 2 min per wash. After application of equilibration buffer to the slides for 5 min, incubation with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP was performed in a humidified chamber for 90 min at 37 °C, using plastic coverslips. Plastic coverslips were used to ensure even staining of the samples. The incubation was stopped by placing the slides in stop wash buffer for 30 min at 37 °C, in a Coplin jar. The slides were washed in 3 changes of phosphate buffered saline for 3 min each wash prior to being incubated with anti-digoxigenin peroxidase conjugated antibody, using plastic coverslips, in a humidified chamber for 30 min at room temperature. Slides were stained in diaminobenzidine (DAB; Research Genetics, Inc, Huntsville, AL) using coverslips for 2 min and counterstained with methylene blue (Sigma) for 1 min in a Coplin jar. The slides were mounted with Crystal/Mount (Biomedda, Foster City, CA). The brown DAB color product, which indicates staining of the free 3'-OH ends that occur as a result of DNA fragmentation, was observed by light microscopy.

Results

Lysosomal Localization and Activity in Glands during Metamorphosis

We determined the localization of lysosomes and baseline levels of AP activity during metamorphosis in glands from whole animals. Glands were isolated from animals at various stages and biochemical values of AP were determined. AP activity started to rise as early as day -2, it leveled off, and peaked at day 3 (Fig. 1). Prior to day 1, the activity reflects primarily the growth of the gland. The histochemical AP data are in agreement with our biochemical AP results. Lysosomes from day –1 labial glands were restricted to basal regions of the cell (Fig. 2). By day 0, lysosomes had migrated from basolateral to lumenal regions (Fig. 2). By day 3 the lysosomes increased in number and filled virtually the entire cytoplasm, and the gland finally disintegrates (Fig. 2). Day 3 represents the peak of apoptosis in the glands based on our biochemical and histochemical AP findings. These data suggest that alterations in lysosomes and lysosomal enzymes are one of the earliest detectable changes that occur in degenerating glands.

Effect of 20HE on Lysosomal Activity in the Labial Gland

Isolated abdomens from ligated day 0 animals were injected with one daily injection of either 20HE or vehicle for various periods. Exposure of labial glands to 20HE increased the levels of AP activity after 8 and 72 h incubations (Fig. 3). The increase at 8 h suggests that the 20HE injection mimics the first endogenous peak of the hormone on day -0.5 (Bollenbacher et al. 1981). The result at 72 h suggests that 20HE induces apoptosis after 3 days of multiple injections. This timeframe, 3 performed the following experiments. A single injection of 20HE or vehicle was administered to abdomens and apoptosis was assessed after 120 h. This incubation period was chosen because the gland dies over a five-day interval during metamorphosis. A single injection of 20HE triggered cell death as indicated by the increase in the number and movement of lysosomes after 120 h by histochemistry (Fig. 4).

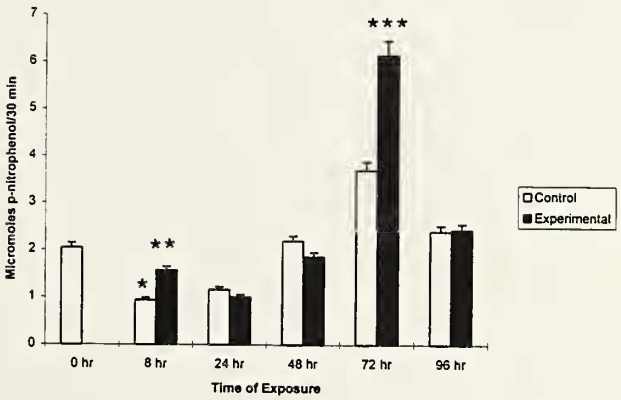

Fig. 3. Effect of 20HE on As Activity in Labial Glands from Abdomens. Animals were ligated at day 0 to remove the source of endogenous 20HE production. Abdomens were injected with 10% ethanol (vehicle; open bars) or 50 mg of 20HE (filled bars). One injection was given for the 8 h and 24 h incubation periods, while one injection every 24 h was administered at the other times. The biochemical As assay was performed on the glands. Values represent means ± SEM of at least three independent experiments. 20HE induces apoptosis in labial glands after 72 h. Single asterisk indicates significant differences from 0 h, $p < 0.03$. Double and triple asterisks indicate significant differences between hormone treated and control glands: **, $p < 0.001$; and ***, $p < 0.004$.

Effect of 20HE on DNA Fragmentation

To determine DNA fragmentation, we assessed the presence of single-strand breaks by TUNEL technique, as illustrated in Figures 5 & 6. Labial glands were isolated from intact metamorphosing animals as well as from isolated abdomens that were injected with vehicle or 20HE. TUNEL staining was barely detectable as the gland enters metamorphosis, day 0 (Fig. 5). An intense signal, however, was detectable at day 4 (Fig. 5). One set of isolated abdomens received multiple injections, one every 24 h, of vehicle or 20HE. The intensity of staining in nuclei from experimental glands was higher than that of controls at 120 h (Fig. 6). However, there were fewer nuclei in 20HE-treated glands. Glands that were exposed to a single 20HE treatment displayed similar TUNEL staining, as did glands that were exposed to multiple 20HE treatments (Fig. 6). In addition, 20HE-treated glands displayed a morphology that was similar to that of dying glands (Fig. 5).

Discussion

There is no doubt that 20HE regulates apoptosis in a variety of insect tissues. However, the precise mechanisms by which the hormone directly or indirectly induces cells to undergo cell death are not yet understood. Like other steroid hormones, 20HE when bound to its receptor, acts as a transcriptional activator. Indeed 20HE regulates several genes that are involved in apoptosis. The three pro-apoptotic genes so far cloned in *Drosophila*, reaper, head involution defective (hid), and grim, are all upregulated by 20HE (White et al. 1994; Grether et al. 1995; Vucic et al. 1997). The upregulation of reaper and hid mRNAs immediately precedes the destruction of the larval salivary glands in *Drosophila* (Dorstyn et al. 1999). These are very encouraging data because these gene products may exist in *Manduca* tissues as well. 20HE should also activate death genes that are responsible for the demise of the labial glands.

The movement of lysosomes from basolateral to apical regions in the cells of the labial gland (Halaby et al. 1994; and this report) suggests that lysosomes play a pivotal role in the destruction of the cell. The lysosomal movement, presumably a result of alterations in cytoskeletal components, specifically microtubules, is currently under investigation. The cytoskeleton has been shown to undergo reorganization during the death of the salivary gland in *Drosophila* (Jochová et al. 1997b). The increase in lysosomal enzyme activity during labial gland degeneration is one of the earliest detectable morphological markers of apoptosis (Zakeri et al. 1993; Halaby et al. 1994). 20HE may directly stimulate acid phosphatase activity in the labial glands as was shown to be the case in Corcyra cephalonica (Ashok & Dutta-Gupta 1988). Lysosomal hydrolases are prominent during the histolysis of insect intersegmental muscles (Lockshin & Williams 1965a,b) and salivary glands (Aidells et al. 1971). Lysosomal enzymes also play pivotal roles in the apoptotic deaths of the mammary gland, prostate gland, and uterus (Helminen & Ericsson 1971; Moulton & Koenig 1983; Sensibar et al. 1990). The selective activation of these hydrolases may be used therapeutically, such as their employment to induce tumor regression of mammary carcinomas (Gullino & Lanzerotti 1972; Cutts 1973), as a means of killing harmful cells while sparing healthy ones.

The DNA fragmentation induced by 20HE in labial gland nuclei was detected later than were the early lysosomal-induced cytoplasmic damage. This pattern of cell death has also been observed in Calliphora salivary gland (Bowen et al. 1993) and mammalian mammary gland (Strange et al. 1992; Tenniswood et al. 1992; Zakeri et al. 1995). Our results indicate that 20HE may promote accelerated apoptosis in the labial gland, but that the TUNEL technique may not be sensitive enough to detect this DNA destruction (Labat-Moleur et al. 1998; Cuello-Carrion & Ciocca 1999). This is presumptively due to the fact that the nuclei in hormone-treated glands are being preferentially degraded, resulting in fewer free 3'-OH ends available for the TdT-catalyzed reaction to occur. Exogenously administered 20HE has been demonstrated to promote the accelerated demise of nuclei from muscle in *Manduca* moths (Hegstrom & Truman 1996; Hegstrom et al. 1998) and nuclei from *Manduca* larval muscles and motoneurones (Weeks & Truman 1986).

Our results indicate that a single injection of 20HE can trigger complete cell death of the labial gland (Figs. 4 & 6). The single exposure of the labial glands to the hormone was sufficient to induce steroid-triggered apototic responses, DNA fragmentation and activation of lysosomes.

Othershave reported, using in vitro experiments, that a brief rather than continuous exposure of insect organs to 20HE resulted in apoptosis of those tissues (Jiang et al. 1997; Streichert et al. 1997). Further research is needed to elucidate the exact mechanism by which 20HE and other steroid hormones cause apoptosis.

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